Annals of Botany **97**: 309–315, 2006
doi:10.1093/aob/mcj051, available online at www.aob.oxfordjournals.org

BOTANICAL BRIEFING

Programmed Cell Death in Floral Organs: How and Why do Flowers Die?

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Received: 4 October 2005 Returned for revision: 12 October 2005 Accepted: 29 November 2005 Published electronically: 4 January 2006

• *Background* Flowers have a species-specific, limited life span with an irreversible programme of senescence, which is largely independent of environmental factors, unlike leaf senescence, which is much more closely linked with external stimuli.
• *Timing* Life span of the whole flower is regulated for ecological and energetic reasons, but the death of individual tissues and cells within the flower is co-ordinated at many levels to ensure correct timing. Some floral cells die selectively during organ development, whereas others are retained until the whole organ dies.
• *Triggers* Pollination is an important floral cell death trigger in many species, and its effects are mediated by the plant growth regulator (PGR) ethylene. In some species ethylene is a major regulator of floral senescence, but in others it plays a very minor role and the co-ordinating signals involved remain elusive. Other PGRs such as cytokinin and brassinosteroids are also important but their role is understood only in some specific systems.
• *Mechanisms* In two floral cell types (the tapetum and the pollen-tube) there is strong evidence for apoptotic-type cell death, similar to that in animal cells. However, in petals there is stronger evidence for an autophagous type of cell death involving endoplasmic reticulum-derived vesicles and the vacuole. Proteases are important, and homologues to animal caspases, key regulators of animal cell death, exist in plants. However, their role is not yet clear.
• *Comparison with Other Organs* There are similarities to cell death in other plant organs, and many of the same genes are up-regulated in both leaf and petal senescence; however, there are also important differences for example in the role of PGRs.
• *Conclusions* Understanding gene regulation may help to understand cell death in floral organs better, but alone it cannot provide all the answers.

Key words: Programmed cell death, flowers, petal, tapetum, pollen-tubes, senescence, ethylene, apoptosis, autophagy, ricinosomes, metacaspases, chromoplasts.

INTRODUCTION: WHAT DO WE MEAN BY PROGRAMMED CELL DEATH IN FLORAL ORGANS?

Recently there has been much controversy over the use of the terms 'senescence' and 'programmed cell death' (PCD), especially with regard to leaves (Thomas *et al*., 2003; van Doorn and Woltering, 2004). In flowers it seems to me that the distinction is largely unnecessary. The deterioration of a flower is certainly programmed, is not a reversible process and inevitably leads to cell death. Thus, I have used the terms essentially interchangeably, using PCD more often when discussing the death of individual cell types, and senescence for whole organs.

WHY DO FLOWERS DIE, AND WHY DO THEY LAST LONGER IN SOME SPECIES THAN OTHERS?

Selective removal of reproductive structures is not unique to plants. Although sperm cells continue to be produced throughout male adult life, 99·9 % of human oocytes are removed by PCD (Tilly, 2001), perhaps ensuring that the costs of female reproduction are tightly regulated to benefit the survival of progeny to adulthood. However, unlike in mammals, both male and female reproductive structures in plants are only retained while they are needed, and are developed *de novo*, in perennial species, the following season. The duration of the flower is species-specific and carefully tailored to its ecological requirements. This is important because firstly the flower can be a substantial sink on the plant's resources, and as such is energetically expensive to maintain beyond its useful life (Ashman and Schoen, 1994). In addition, its architecture has been exploited by pathogens that use the stigma as a point of entry, and thus the flower poses an added risk of pathogen attack (Shykoff *et al*., 1996). Another important reason for floral death after pollination is to remove it from the population so that it does not compete for pollinators with the remaining blooms. One of the key triggers for petal death is pollination, which initiates a series of physiological events, orchestrated by plant growth regulators (PGRs). Ethylene is a clear regulator of petal senescence in some species (Stead and van Doorn, 1994); however, in other species including lilies such as *Hemerocallis* (daylilies) and *Alstroemeria* it appears to play little or no part (Woltering and van Doorn, 1988; Wagstaff *et al*., 2005). How petal senescence in these species is triggered and orchestrated remains unknown. Given the failure to find a common regulator for these species, and their taxonomic diversity, it seems likely that several inter-related mechanisms may be at play. Resource allocation has been one trigger proposed, and indeed removal of lower flowers in a cyme can lead to increased longevity of the first flower (Chanasut *et al*., 2003). However, this is clearly not a full explanation for all ethylene-insensitive species.

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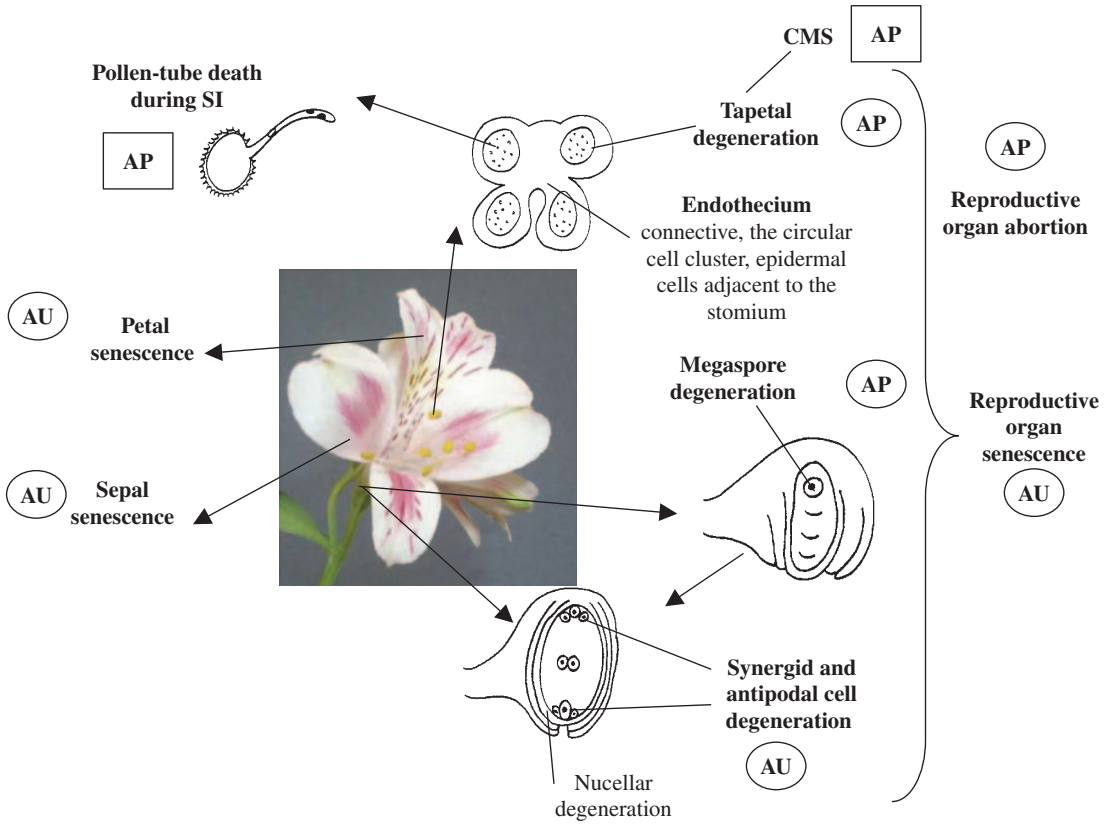

FIG. 1. Sites of programmed cell death in floral organs. SI, self-incompatibility; CMS, cytoplasmic male sterility; AU, autophagous-like mechanism; AP, apoptotic-like mechanism; square indicates strong evidence, circle indicates weaker evidence. Bold text indicates cells and tissues discussed in the text.

An important feature of floral death is that the different floral organs play very different roles. Hence, their life span needs to be appropriately co-ordinated. Likewise, the purpose and fate of the dying cells depends on the organ and tissues involved. At a whole organ level, petals, anthers and stigma are no longer required following pollination, whereas the ovary will mature to contain the developing seeds. In many species there is also a mechanism for rescuing resources from the degenerating organs such as petals, and diverting them to other parts of the plant such as the developing ovary (Stead and van Doorn, 1994). At a tissue and cell level, the situation is even more complex as there is a requirement for some reproductive tissues and cells to die to ensure correct development. For example, the tapetum must degenerate for pollen to develop properly, and synergid cells must die to allow fertilization. However, the fate of the dead cells is very different. In the case of the tapetum, cell contents are used to form the coat of the pollen grains, whereas removal of synergid cells is required for fertilization to occur (Christensen *et al.,* 2002). Some types of cell death in floral organs also depend on specific genetic interactions. PCD occurs as a result of incompatible pollination events (Thomas and Franklin-Tong, 2004), and also as a result of defects in pollen development displayed in cytoplasmic male sterile lines (Balk and Leaver, 2001). Thus, important questions with regard to cell death in reproductive organs are: How do the cells perceive and respond to death signals, or, put more teleologically, how do they know when to die? Are the primary signals processed in the same way by the different organs and cells? Is the type of PCD in floral organs also found in other plant tissues and organs? I restrict myself here to considering the organs that make up the mature flower (Fig. 1). Although fruit ripening and seed maturation include further examples of PCD, these will not be considered here.

HOW DO THE CELLS KNOW WHEN TO DIE?

In some species pollination dramatically shortens floral life span. For example, orchid flowers will last several months but senesce rapidly once pollinated. In several species, including *Petunia*, tobacco, carnation and orchids, senescence is mediated by the evolution of ethylene following contact between pollen and the stigmatal surface, which precedes fertilization (O'Neill, 1997). However, the exact nature of the primary signal resulting in ethylene evolution has not been established, although other PGRs and low-molecular-weight compounds have been implicated (O'Neill, 1997). In carnations, ethylene produced from the pollinated stigma is translocated, via the style and ovary, to the petals. Here it up-regulates ethylene biosynthetic genes and induces the production of ethylene in

the petals (ten Have and Woltering, 1997). Once initiated, the evolution of ethylene becomes autocatalytic (Woodson and Lawton, 1988). This strongly suggests that promoters of the ethylene biosynthetic genes respond to ethylene and contain ethylene-responsive elements (EREs). To date, this has not been verified although an ERE from a senescence- and ethylene-regulated gene in carnation bears similarities to the ERE from an ethylene-responsive fruit-ripening gene, *E4*, suggesting commonality of transcription factors in these two processes (Deikman, 1997).

The response to ethylene is regulated by the production of ethylene receptors but how this regulation is achieved is not clear. In tomato an *ETR1* ('ethylene-resistant')-type ethylene receptor was not transcribed in young flowers or senescent flowers, but only in mature flowers (Payton *et al*., 1996). Furthermore, ethylene receptor expression may itself be regulated by ethylene production. In pea, transcripts of an *ERS* ('ethylene response sensor')-type ethylene receptor were reduced when un-pollinated flowers were treated with an inhibitor of ethylene biosynthesis (Orzaez *et al*., 1999). So the balance between receptor production and ethylene sensitivity is clearly regulated at several levels.

Notably, in species in which ethylene is a major regulator, ethylene-independent signals are also present. Disruption of ethylene signalling or biosynthesis in carnation and petunia results in delayed floral death, but the flowers do eventually die (Michael *et al*., 1993). Perhaps it is these endogenous signals that are active in species where ethylene is not a major regulator. Several global transcriptomic studies (e.g. *Alstroemeria*: Breeze *et al*., 2004; *Iris*: van Doorn *et al*., 2003) have attempted to reveal the genes or pathways regulating floral degeneration in these species; however, no clear patterns have yet emerged. Verifying the role of genes in these species is hampered by the lack of genome sequences and, often, lack of efficient transformation systems. Another possibility is that senescence and PCD are regulated post-transcriptionally, as argued by Thomas *et al*. (2003). Perhaps a complex network of both transcriptional and post-transcriptional control is involved, as is found in other fundamental cellular processes such as the cell cycle. If the underlying ethylene-independent life span control in ethylene-sensitive species is common to ethylene-insensitive species, then models such as *Arabidopsis* and *Brassica* or tomato and petunia may offer better species in which to investigate these control networks. This would be a neat solution to a difficult problem. Langston *et al*. (2005) used this approach to study DNA fragmentation in petunia, showing that the ethylene induction of a 43-kDa nuclease (PhNUC1) was delayed in 35S:*etr1-1* plants but not eliminated. Nine thousand expressed sequence tags (ESTs) have been recently generated from a global transcriptomic analysis of petunia floral senescence (D. Clarke, University of Florida, pers. comm.) and it will be interesting to see what proportion of these genes are up-regulated in 35S:*etr1-1* lines. Likewise, in *Arabidopsis*, transcriptomic and perhaps proteomic analysis of petal senescence in *etr1-1* lines may be a fruitful line of enquiry. However, if ethylene-independent regulation turns out to be species-specific then it is important to continue work with the diverse species currently being studied to appreciate the range of networks employed.

Another important question is whether the same signal differentially regulates PCD in floral organs. In some cases the answer is yes: for example in tobacco, ethylene regulates petal senescence (Rieu *et al*., 2003); however, at the same time ovary tissues continue to develop. So how is a primary signal such as ethylene transduced to ensure the co-ordinated life and death of different floral organs? Presumably this is through differential signal translocation or differential signal perception. Petal margins often degenerate before the centre and cross-sections of developing petals reveal that while the epidermal cells are still functional, mesophyll cells have largely degenerated even before the flower is fully open (Wagstaff *et al*., 2003). So is there a gradient here of a diffusible signal, or of receptors or other intracellular mediators of the cell-death signal? In some cases this signalling differential is very distinct: in the *Arabidopsis gfa2* mutant, synergids fail to undergo PCD, but antipodal PCD is not affected (Christensen *et al*., 2002).

Ethylene is not the only PGR stimulating PCD in floral organs: some links to other PGRs are reviewed in Wu and Cheung (2000). Mutation of gibberellic acid biosynthetic genes *anther ear 1* and *dwarf* results in failure to abort stamens on maize female flowers. Mutation of a gene associated with brassinosteroids (*TS*) ('tasselseed') results in feminization of male flowers, and application of jasmonate (JA) enhances petal senescence in some species (Porat and Halevy, 1993), although this effect may be indirect, through ethylene signalling (Stead *et al*., in press). Elevating cytokinin levels in petunia delayed flower senescence; however, this may also be indirect through changes in sugar transport (Lara *et al*., 2004). So are all these PGRs involved in floral PCD in all species? Or are there important quantitative or even qualitative species-specific differences in their effects? Perhaps metabolomic approaches to measure endogenous levels of PGRs, coupled with a more extensive use of mutants, may begin to address these questions.

IS THERE JUST ONE PCD MECHANISM OPERATING IN FLORAL ORGANS?

van Doorn and Woltering (2005) have recently categorized plant PCD into three types: apoptotic, autophagic and neither apoptotic nor autophagic. In animal cells four types of apoptosis have been described (Orrenius *et al*., 2003), three of which involve cytochrome *c* release from the mitochondrion controlled by a family of proteins (Bcl-2) that interact with the mitochondrial membrane to facilitate or inhibit this process. Cytochrome *c* then activates a family of *c*ysteine *asp*artate-specific prote*ases* (caspases), which both regulate and effect PCD. Apoptotic PCD in animals is characterized by cytological features, including chromatin and nuclear condensation and marginalization followed by DNA fragmentation into nucleosomal units known as DNA laddering, nuclear blebbing and formation of membrane inclusions known as apoptotic

bodies (Cohen, 1993). The apoptotic bodies are then engulfed by neighbouring living cells.

In the tapetum and pollen-tubes, there is compelling evidence to support an important role for the mitochondrion and involvement of caspases. This suggests a mechanism similar to animal apoptosis, although caution must be exercised in drawing too close a parallel, as engulfment of cellular remains by other cells does not occur in plants (van Doorn and Woltering, 2005). Following its nutritive role during pollen development, the tapetum degenerates. This is characterized by chromatin condensation in *Lobivia rauschii* and *Tillandsia albida* (Papini *et al*., 1999), and by DNA fragmentation in barley anthers (Wang *et al*., 1999). In *Brassica oleracea*, *Brassica napus*, *Digitalis purpurea* and a cultivated form of *Fuchsia* there is also nuclear blebbing (A. D. Stead, Royal Holloway, University of London, unpubl. data). Furthermore, in *PET1* cytoplasmic male sterility (CMS) of sunflower, there is cytochrome *c* release into the cytosol followed by changes in cell morphology, loss of outer mitochondrial membrane integrity and a fall in the respiratory control ratio (Balk and Leaver, 2001). Assuming that CMS is just anticipating a normal event (quite a major assumption), it might be concluded that PCD in the tapetum is apoptotic; however, we still do not know how it is triggered. Studies of nuclear mutations resulting in tapetal degeneration may be helpful: morphological changes were charted in a rice male sterile mutant (Ku *et al*., 2003), including cytoplasmic shrinkage, membrane blebbing, vacuolation, changes in mitochondrial morphology and early DNA fragmentation. However, cytochrome *c* leakage and respiratory control ratio were not measured in this system. Further studies on PCD in non-CMS tapetal cells would be helpful in this context.

Another example of apoptotic-like PCD in floral organs is in the death of the pollen-tube during self-incompatible pollination interactions. In *Papaver*, Thomas and Franklin-Tong (2004) showed that self-incompatibility (SI) stimulated increases in cytosolic [Ca^{2+}], which in turn activated release of cytochrome *c* into the cytosol and induced caspase-3-like activity. The caspase-3-like activity cleaved poly(ADP-ribose) polymerase (PARP), a classic substrate for caspase-3 enzymes, and was also inhibited by the peptide Ac-DEVD-CHO, which blocked DNA fragmentation and pollen-tube growth.

Another form of animal PCD is autophagy, in which vesicles (autophagosomes) containing proteins and organelles are transported to the hydrolase-packed lysosome. Here the contents are digested to generate monomeric building blocks (Klionsky and Emr, 2000). Evidence for autophagic PCD in petals comes from the identification of organelles that deliver proteases to the vacuole. The hypothesis is that the vacuole may be playing a homologous role to the animal lysosome. Healthy epidermal *Arabidopsis* leaf cells contain plant-specific, endoplasmic reticulum (ER)-derived compartments containing precursors of cysteine proteinases known as protease precursor vesicles (PPVs) (Hayashi *et al*., 2001). On application of stress, the PPVs fuse with each other and with the vacuole, delivering protease precursors to the vacuole, which then effect the maturation of other proteases and participate in the disassembly of cellular components during senescence. One senescence-induced cysteine protease found in *Arabidopsis* PPVs is vacuolar processing enzyme-γ (VPE γ). The PPVs deliver VPE γ to the vacuole where it is required for the processing of a number of proteins. VPEs are one of the two groups of plant proteases which are candidates for plant caspases, the other being metacaspases (Sanmartín *et al*., 2005). Modelling of VPEs shows tertiary structure homology to caspases (Sanmartin *et al*., 2005), with VPE γ being closest in structure to caspase-8. VPE γ also shows caspase-1 activity and binds to caspase-1-specific inhibitors (Rojo *et al*., 2004). VPEs are up-regulated during leaf and cotyledon senescence, stress (Kinoshita *et al*., 1999), and during pathogen defence (Rojo *et al*., 2004). So is this mechanism active in floral organs? Papain-class proteases identified from fruit, senescent petals and ovaries, and degenerating endosperm all contain a conserved Asn residue in the protein precursor that is probably a target for cleavage, resulting in maturation of the enzyme (Kinoshita *et al*., 1999). As VPEs target Asn residues, VPEs may activate these proteases within the vacuole of senescent tissues. Bringing together the different strands of evidence, there seems to me to be a good argument for VPEs performing a regulatory role in many if not all the forms of PCD seen in floral organs.

Similar organelles to PPVs have been isolated from *Ricinus communis* endosperm known as ricinosomes. These organelles develop as the cells undergo PCD, and rupture releasing their cargo of proteases directly into the cytosol. Acidification triggers ricinosome rupture; thus, release of proteases from ricinosomes may be activated after acidification of the cytosol following vacuolar leakage (Schmid *et al*., 1999). Ricinosomes contain CysEP, a type of cysteine endoprotease with a C-terminal KDEL motif (Gietl *et al*., 1997), which directs proteins to the ER lumen. KDEL-containing cysteine proteases have been identified in a number of senescent floral organs including *Hemerocallis* (daylily) petals (Valpuesta *et al*., 1995) and in *Pisum sativum* senescent ovaries (Cercos *et al*., 1999). In senescent daylily petals the KDEL-containing protease is within vesicles similar to ricinosomes (Schmid *et al*., 1999) so ricinosome-mediated PCD may be a feature of petal PCD. However, ricinosome-like vesicles are clearly distinct from the PPVs in that they are presumed to act downstream of the vacuolar leakage, and to deliver their cargo into the cytosol rather than the vacuole (Fig. 2). At least two metacaspases (*AtMCP2f* and *AtMCP1a*) are also thought, based on array data, to be induced in senescing flowers (Sanmartín *et al*., 2005), but their location is unknown. Confirmation of their role *in planta* will be important.

DO FLORAL ORGANS AND OTHER PLANT TISSUES DIE IN THE SAME WAY?

A functional categorization of PCD can be made on the basis of the fate of the cell contents. Remobilization is central to leaf, sepal and petal senescence (Thomas *et al*., 2003) and in a different way also to tapetal PCD. But

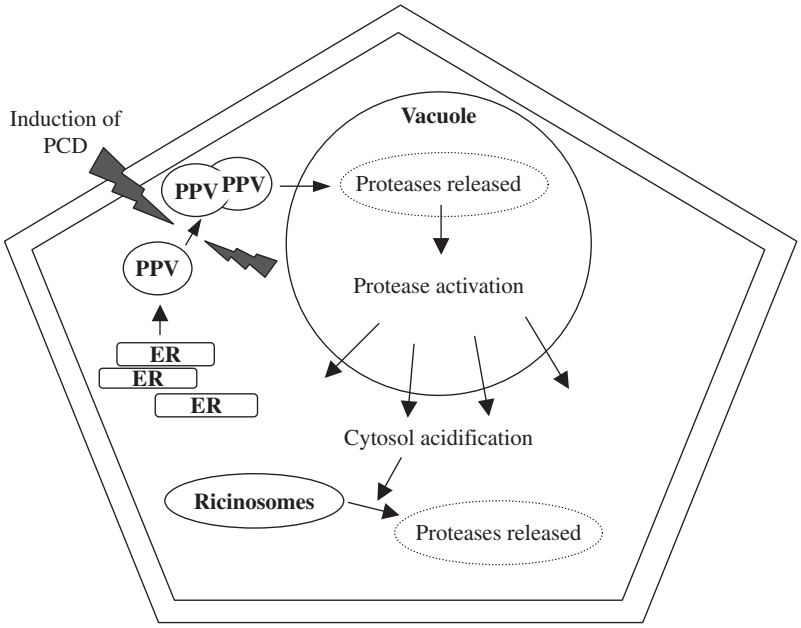

FIG. 2. Tentative model for the role of protease precursor vesicles (PPVs) and ricinosomes in cell death. Derived from the endoplamsic reticulum (ER), PPVs fuse with each other and then the vacuole when programmed cell death (PCD) is induced (by extracellular or intracellular signals). Proteases are released from the PPVs, which activate further proteases leading to vacuolar membrane leakage. Consequent acidification of the cytosol induces ricinosome breakdown, releasing further proteases which degrade cytosolic proteins.

endothecium, synergid, antipodal cell or pollen-tube PCD is the selective death of unwanted cells.

In green tissues, the chloroplast is seen by some (Thomas *et al*., 2003) as the key participant in the senescence process, and an early sign of senescence in green tissues is conversion of chloroplasts to gerontoplasts. Sepals, the floral organ that most closely resembles leaves, senesce in a similar way: in broccoli, sepal chlorophyll degradation is the first visual sign of senescence (Page *et al*., 2001). Petals are, however, not usually green, and an early step in their development is a conversion of chloroplasts to chromoplasts. This conversion has been compared with the chromoplast/gerontoplast transition (Thomas *et al*., 2003) with the inference that petals are most similar to senescent leaves. This agrees with the very early cell death seen in flowers (Wagstaff *et al*., 2003) presumably associated with nutrient remobilization. However*, in-silico* comparison of transcriptome changes in senescent *Arabidopsis* leaves and petals indicates that 25–30 % of genes share similar patterns of expression (Stead *et al*., in press). A comparison of transcriptome changes during petal senescence and fruit ripening would be interesting. Complex networks of ethylene, JA and salicylic acid operate in leaves (He *et al*., 2001), which may differ from those in petals, as ethylene does not have the same dramatic effects on leaves seen in petals (Grbić and Bleeker, 1995). At a subcellular level morphological changes to subcellular compartments during PCD are shared by many different cell types and tissues (Rogers, 2005). VPEs are found in leaves, roots and flowers; ricinosomes are seen in seed and petal tissues; and caspase activity is detected in pollen-tubes undergoing SI, and in many non-floral tissues during natural senescence and also during pathogen responses (Sanmartín *et al*., 2005).

CONCLUSIONS: WHAT DO WE REALLY WANT TO KNOW?

Progress in our understanding of PCD in plants has been rapid in the last 10 years, but the key regulators of some types of floral organ senescence, such as petal senescence in ethylene-insensitive species, remain obscure. It is also unclear whether regulation of petal senescence and PCD in these species is similar or divergent. The latter is an important question to resolve before a good model for these species can be developed. Even in ethylene-regulated petal senescence, the primary signal initiating the ethylene cascade remains elusive and needs pinning down, and again it is not at all certain that it will prove to be common to all species. Work on leaf senescence is suggesting that the search for a 'master-switch' gene or genes of plant senescence may be futile, and that a complex network of endogenous and exogenous signals tips the balance towards death. Floral senescence appears much more tightly regulated developmentally, so should we be looking for a master regulator here? Transcriptome analyses have not revealed any obvious candidates, but perhaps we should not stop looking yet. Another area that seems under-researched is our understanding of the promoters of floral-senescence genes. Genomic approaches and new developments in bio-informatics are powerful tools for developing this area and exploring how far transcription factors are shared between leaf senescence, floral senescence, fruit ripening and localized PCD. This will help to reveal the upstream regulatory networks, even if a master-switch is not the answer. Proteomics and metabolomics may also help to understand post-transcriptional regulatory networks, and define better the role of PGRs. Transgenics and mutants

TABLE 1. *Comparison of signalling and possible mechanisms of programmed cell death (PCD) in floral organs*

Floral Organ	Intercellular signals	Intracellular signals and possible mechanisms for PCD	References
Sex organ abortion	GA and brassinosteroids	?	Wu and Cheung (2000)
Tapetum in cytoplasmic male sterility lines	Mitochondrial dysfunction	Cytochrome *c* release, followed by loss of mitochondrial function	Balk and Leaver (2001)
Synergids	Pollination in some species	Requires mitochondrial function?	Christensen *et al*., 2002
Petal senescence	Ethylene in some species	Ca^{2+}/phosphatase signalling, reactive oxygen species increases	Porat and Halevy (1993), Kinoshita *et al*. (1999), Orzàez *et al*. (1999), Schmid *et al*. (1999), Wagstaff *et al*. (2003), Lara *et al*. (2004)
	Jasmonate (via ethylene?)	Activation of vacuolar lytic enzymes through vacuolar processing enzyme (caspase-1 activity)	
	Cytokinin (via sugar transport)	Activation of vesicle-bound proteases following vacuolar leakage	
Pollen-tube	During self-incompatiblity interactions	Increased Ca^{2+}, resulting in cytochrome *c* release and caspase-3 activity	Thomas and Franklin-Tong (2004)

are powerful tools, and perturbation of specific pathways such as manipulation of PGR levels using inducible promoters will also contribute to our understanding of the roles of PGRs in floral senescence and PCD. At a cellular level plant PCD seems poised on the edge of major advances in unravelling protease cascades and intracellular signalling events. We can start to build a picture of the types of mechanisms operating in different cell types (Fig. 1, Table 1); however, it is far from complete. So we are still some way from a systems biology approach that might describe the whole process.

ACKNOWLEDGEMENTS

Many thanks to Carol Wagstaff and Tony Stead for their many helpful comments on the manuscript.

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